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Bioremediation of Crude Oil Polluted Soil using Biofertilizer from Nitrogen-Fixing and Phosphate-Solubilizing Bacteria

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Abstract: This study examined bioremediation of crude oil polluted soil (PS) stimulated with nitrogen-fixing bacteria (NFB) and phosphate-solubilizing bacteria (PSB). Five set-ups designated as A (500 g PS + 50 g NFB); B (500 g PS + 50 g PSB); C (500 g PS + 50 g NFB+PSB); D (500 g PS + 50 g NPK); and E (500 g PS only: control) were designed. Total petroleum hydrocarbons (TPHs) were monitored for 4 weeks. Toxicity of the biofertilizer on maize plant was determined. The combination of the biofertilizers with normal soil for the ecotoxicity testing was in the following ratios: 100:0, 75:25, 50:50, 25:75, and 0:100. The NFB were classified as *Azotobacter* sp. and *Rhizobium* sp. while the PSB identifies as *Pseudomonas* and *Bacillus* using their 16S rRNA gene sequences and deposited in GenBank under the accession numbers MN134485.1-MN134488.1. After 28 days study, TPH reductions were 97.8%, 97.5%, 94.3%, 92.1%, and 34.6% in NFB, NFB+PSB, PSB, NPK treatments, and control. There was significant difference ($P<0.05$) between the set-ups when compared to the control. For toxicity testing, the 25:75 concentration in all treatment set-ups best supported plant growth. It was concluded that biofertilizer is effective in remediating oil contaminated soil and in improving soil fertility.

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Keywords: bioremediation, nitrogen-fixing bacteria, phosphate-solubilizing bacteria.

1. Introduction

Biofertilizers are microbial inoculants which are artificially increased cultures of certain soil microorganisms that can enhance soil fertility and crop productivity [1,2] Biofertilizers are used to stimulate indigenous hydrocarbon degrading bacteria and fungi during bioremediation [3, 4]. Nitrogen-fixing bacteria enrich the soil nutrient from oil killed microorganism and the soil itself. The bacterial genera are *Clostridium, Rhizobium, Azotobacter, Azospirillum* and *Beijerinkia* [5].

Phosphate-solubilizing bacteria can be employed for the production of biofertilizers which actually improve the nutrient quality of soil [6]. Examples of the bacterial genera are *Pseudomonas, Bacillus, Flavobacteria, Aspergillus, Agrobacterium, Micrococcus, Achromobacter* [7]. Nitrogen-fixing bacteria release the nitrogenase enzyme system which enhances bioremediation process of crude oil polluted soil [5]. Illegal refining of crude oil and other petroleum operations in Ogoniland have negatively impacted on agricultural activities such as farming and fishing, thereby increasing poverty in the region.

The use of inorganic fertilizer (e.g. NPK) to enhance bioremediation, poses environmental and ecological challenges [8]. There is need to employ remediation techniques that will restore contaminated media (e.g soil) to a state that can be used for agricultural and other important activities. The use of biofertilizer reduces the rate of environmental pollution. Commercial feasibility of biofertilizer production could have a price fall effects on chemical fertilizer. The processes facilitate soil enrichment and promote plants' growth. It is also a sustainable and environmentally friendly approach to the remediation of hydrocarbon polluted media. It increases microbial populations in the soil by enhancing hydrocarbon utilizing bacteria. This research, therefore, seeks to investigate the use of nitrogen-fixing and phosphate-solubilizing bacteria for bioremediation of crude oil polluted soil in Kegbara-Dere (K-Dere), Gokana Local Go vernment Area of Rivers State and to further determine the toxicity level of the biofertilizer that support remediated soil.

2. Materials and Methods

2.1 Sample Collection

The polluted soil was sourced from Barabeedom eastern zone of Kegbara-Dere (K-Dere) in Ogoni-land which is located close to Bomu manifold flow station in connection with Trans-Niger pipeline, which transports crude oil through Ogoniland to Bonny trunk line. The nearest farmland and river are polluted because of oil spill from pipeline leakage. Co-ordinates of sampled points were determined using Global Positioning System (GPS).

The co-ordinates of sampled point are: Latitude: 4.671768, Longitude: 7.253275 and point B are: latitude: 4.675735, Longitude: 7.251268. Soil samples were source with aid of soil auger from polluted areas at Barabeedom of K-Dere, Gokana L.G.A. Five random samples (500 g) each was collected at depth of 0-15 cm and another five samples were collected at depth of 15-30 cm and sieved through a 4.5 mm mesh sieve. The ten soil samples were homogenized to make a composite sample then packaged in sterile container and immediately transported to laboratory. The test seed (maize) were collected in polythene bags from rural farmers.

2.2 Microcosm Set-up

Approximately 1.5 kg of soil was placed in plastic vessel labeled A, B; C and D (Table 1). The 150 g polluted soil (PS) containing 10 % crude oil was left undisturbed for 48 h. After these time, 10 % of each bio-fertilizer namely Nitrogen-fixing bacteria (NFB), Phosphate-solubilizing bacteria (PSB) and NFB + PSB were supplemented into each oil-polluted soil labeled A, B and C and mixed. Vessel D served as control with only soil and crude oil. Also, additional control (comprising autoclaved soil and 0.5 % NaN_3) in vessel D1 was placed to observe degradation process of crude oil in soil arising from non-biological factors.

The moisture was attuned to 60 % by adding water, and content were tilled for aeration 3 times every week for 4 weeks. The plastic vessel was then hatched at 30±2°C in three places. Samples collected were moved to laboratory for analyses within 2 weeks time interval (14th and 28th day). Bioremediation for hydrocarbons in different experiments were monitored using standard recommended methods of analysis [9]. Table 1 show the experimental design.

Table 1: Experiment Design

Set-up	Treatment Content
Set A	500 g PS (autoclaved) + 50 g NFB (10 %)
Set B	500 g PS (autoclaved) + 50 g PSB (10 %)
Set C	500 g PS (autoclaved) + 50 g NFB (10 %) + PSB (10 %)
Set D (Control)	500 g PS (autoclaved) + 50 g N.P.K
Set D_1	500 g PS (autoclaved) + no amendment

PS: Polluted soil, **NFB:** Nitrogen-fixing bacteria, **PSB:** Phosphate-solubilizing bacteria.

2.3 Toxicity Test

A total of fifteen polythene vessels were employed. The set-up for each test-plant included one positive control (normal soil) and four vessels of varying concentrations of the biofertilizer. Each's polythene vessel was contained specific quantity of soil and the respective quantity of the bio-fertilizers in ratio: 4:0; 3:1; 2:2; 1:3 and 0:4. For each of these test-plants, experiment was conducted with various bio-fertilizers (phosphate-solubilizing bacterial bio-fertilizer, Nitrogen-fixing bacterial bio-fertilizer, and mixture of phosphate-solubilizing and Nitrogen-fixing bacterial). The experiment took 14 days (2 weeks) and plants monitored for seed germination, leaf coloration and root elongation.

2.4 Enumeration of Microorganisms

Soil slurry was prepared and used for the preparation of a 10-fold serial dilution by mixing 1g of wet soil with 9 ml of sterile physiological saline suspension in a test tube. Subsequently serial dilution from that test tube was performed starting from 10^{-1} to obtain 10^{-7} dilution. Determinations of counts of the various physiological groups of bacteria were carried out in triplicates and counts obtained expressed as colony forming units per gram (CFU/g) of soil.

2.4.1 Enumeration of Total Heterotrophic Bacteria

Total culturable heterotrophic bacteria in soil were enumerated on nutrient agar which comprised the following: meat extract 1g, yeast extract 2g; peptone 5g, NaCl 2 5g, agar No. 2 powder 15g, and distilled water 1litre. The final pH was 7.4±0.2. Nitrogen-fixing bacteria were isolated from the soil samples using the method employed by Ogugbue *et al.* [10]

2.4.2 Enumeration of Phosphate-Solubilizing Bacteria

The prepared Pikovskaya medium for isolation was sterilized and autoclaved using temperature at 121° C for fifteen minutes. The medium was placed in petri-plates and given time to solidify.About0.1 millimeter of polluted soil solution was spread on plate by spread-plate technique. The plates are then incubated in 5 to 7 days at 37°C [11]. After 5 to 7 days incubation resulted in growths. Dilution 10^{-3} and 10^{-4} were selected for screening halo zone formation around colonies. Screening of phosphate-solubilizing bacteria was carried out using bromocresol green as indicator for dye utilized for introductory is screening and gestation done at 37 °C for 12 days.

The green color zone that produce isolates were specified as phosphate-solubilizes in solid culture conditions. The obvious halo zone was evaluated by withholding their colony diameter. To ascertain whether these isolates were phosphate-solubilizers they were tested for their acetylene reduction activity assay in liquid culture.

2.4.3 Enumeration of Nitrogen-Fixing Bacteria

Individual nitrogen-fixing microbes was separated or isolated by spread plating on nitrogen free enrichment media. Exactly 0.5 mL part of these samples was pipetted and plated on solid medium. Glass spreader was sterilized using alcohol and flamed before using then in spreading the inoculums on the plates and were incubated at room temperature, purity was accomplished by sub-culturing continually on nutrient agar which was prepared by dissolving 2.8g nutrient agar in 100 mL distilled water then autoclaved in 121° C within fifteen minutes.

Screening for nitrogen-fixing bacteria was done using nitrogen free malate media [12], containing bromothymol blue as an indicator was used in primary screening and incubation at 37 °C up to 24 h**.** Blue color area producing isolates were specified as nitrogen fixers in solid culture conditions. The colouring area was evaluated by deducting colony size from colouring zone size. To ascertain whether these isolates are nitrogen fixers, they were also tested for "acetylene reduction activity assay" in liquid culture.

2.4.4 Enumeration of Hydrocarbon Utilizing Bacteria

Hydrocarbon utilizing bacteria in soil samples were enumerated using a modified mineral salt medium of Mills. It contained: $MgSO_4.7H_2O$ 0.40g; KCl, 0.28g; KH_2PO_4 0.80g; Na_2HPO_4 1.20g; NH_4NO_3 0.40g; NaCl 15g and agar No. 2 powder 20 g, all in 1 liter of de-ionized water. The pH of the medium was adjusted to 7.1 and subsequently sterilized at 121 °C for 15 min.

Crude oil was introduced to the medium through vapour phase transfer by soaking a 9cm Whatman No. 1 filter paper with 10 ml of fresh Bonny light crude oil. The flooded filter paper was then placed on the lid of the agar plate and incubated for 7 days at 25±8 °C in an inverted position [13]. The filter papers served as a source of energy and carbon and supplied the hydrocarbons by vapour-phase transfer to inverted inoculums.

2.5 Characterization of Isolates

Colonies of nitrogen-fixing bacteria and hydrocarbon utilizing bacteria were picked randomly using a sterile inoculating wire loop and purified by sub-culturing on nutrient agar plates. The plates were incubated at 28±2 °C for 24 h to obtain pure colonies. Gram reaction, cell arrangement, colonial morphology and biochemical characteristics of purified colonies were examined. Gram-negative, grayish, mucoid and flat colonies with a pear-shaped suggestive of *Nitrobacter* were picked and identified with reference to Bergey's Manual of Systematic Bacteriology by Holt *et al.* [14]. The physicochemical properties were also used to characterize the isolates. The total organic carbon, moisture content, phosphate, sulphate and nitrate contents were determined using standard methods. The pH was analysed with pH meter (Jenway 3015) and the residual total petroleum hydrocarbon in the soil was determined using a modified EPA 8015 technique. All analyses were carried out in triplicates.

2.6 Statistical Analysis

At the end of this research, the data generated were compared with result from positive control soil [15, 16]. The data sourced from this analysis are analyzed using SPSS version 20.0 for one-way ANOVA to ascertain significant difference between mean values at $P < 0.05$ and correlation coefficient.

3. Results

The changes in population of the various physiological groups of bacteria in the treated soil during the study period are as presented in Table 2, Figs 1—17 and Plates 1—7 respectively. The baseline microbiological and physiochemical properties of the polluted soil (PS) are shown in Table 2. TPH and THC in these un-modified polluted soils were 8987.5742 ±0.00 and 6000± 0.00 mg/kg respectively. pH was observed as acidic at pH 6.5 ±0.169. Temperature was 28 ±0.6 °C; moisture, 0.16 ±0.01 %. Electrical conductivity was 3.6 ±0.25 µS/cm while nitrate, phosphate and sulphate contents were 86.2 ±0.35 mg/kg, 34.8 ±0.7 mg/kg and 24.1 ±0.5 mg/kg, respectively.

The baseline analyses for microbiological parameters (Total heterotrophic bacteria (THB), hydrocarbons using bacteria (HUB) and nitrogen fixing bacteria (NFB) showed that THB had count of 1.58×10^7±0.205 while HUB and NFB counts were 7.9×10^4 ±0.170 and 7.4×10^4±0.162 CFU/g respectively. Fig. 1: THBC was 7.12 Log_{10}cfu/g in Site A while in Site B it was 5.82 Log_{10}cfu/g. HUBC was 4.7 and 4.9 Log_{10}cfu/g in site A (Artisanal refinery site) and B (Polluted farmland) respectively. TABC was fairly the same in both samples (5.12 and 5.25 Log_{10} cfu/g).

NFBC was 4.87 and 4.209 Log_{10}cfu/g while the PSBC was 4.73 and 4.88 Log_{10}cfu/gin Site A and Site B respectively. The result presented in Table 3 revealed the compositions of the bio-fertilizer after Nitrate concentration were noticed as 6.27 mg/kg, Phosphate was 0.029 mg/kg. pH and Temperature were 8.5 and 28 °C while total nitrogen and organic matter were 15.6 mg/kg and 40 mg/kg respectively. Table 2 is a summary of all the physicochemical and microbiological parameters of crude oil contaminated soil before bioremediation. The pH of experimental set up modified using NFB varied slightly from 7.52 to7.41 on 14th day and finally 7.24 on 28th day while pH of PSB-modified reduced from 7.45 to 7.38 on 14th day and finally to 7.21. NFB+PSB amended samples had decreased pH from 7.84 to 6.85 on 28th day. However, control set up had decreased pH from 6.54 to 4.81.

Table 2: Physicochemical and Microbiological parameters of crude oil contaminated soil before bioremediation

Parameters	Unit	Values ± S.D
Temperature	°C	28 ±0.6
Nitrate	mg/kg	86.2 ±0.35
Phosphate	mg/kg	34.8 ±0.7
Sulphate	mg/kg	24.1 ±0.5
Moisture	%	0.16 ±0.01
pH		6.5 ±0.169
TOC	%	14.18 ±0.337
THC	mg/kg	6000 ±0.00
TPH	mg/kg	8987.5742 ±0.00
TH	CFU/g	$1.58 \times 10^7 \pm 0.205$
HUB	CFU/g	$7.9 \times 10^4 \pm 0.170$
NFB	CFU/g	$7.4 \times 10^4 \pm 0.162$

Results are expressed as mean ± standard deviation

The result presented in Fig. 3 revealed a decline in nitrate concentration during bioremediation study. Firstly, the set up modified using NPK revealed decline in nitrate concentration from 252.91 mg/kg on zero day to 127.12 mg/kg on 14th day then 70.53 mg/kg.

In experiment modified using nitrogen- fixing and phosphorous solubilizing bacterial suspension, nitrate content decreased from 143.91 mg/kg on initial day to 127.12 mg/kg on the 14th day and further declined to 18.99 mg/kg. In set up modified using phosphate solubilizing bacterial suspension, the nitrate content dropped from 164.18 mg/kg on initial day to 69.03 mg/kg then to 30.43 mg/kg on 28th day. The result shown in Fig. 4 describes alteration in pH content of the treatments. The results presented in Fig. 5 revealed 293.46 mg/kg on the initial day and then 134.05 mg/kg on 14th day and 70.468 mg/kg on 28th day.

PSB-amended sample had total nitrogen concentration of 180.09 mg/kg. Samples amended with NFB+PSB declined from 561.36 mg/kg to 310.05 mg/kg on day 14 then to 83.16 mg/kg on 28th day. NPK-amended samples increased from 168.05 mg/kg to 199.07 mg/kg. The control had value of 27.9 mg/kg initially and 29.2 mg/kg on 14th day.

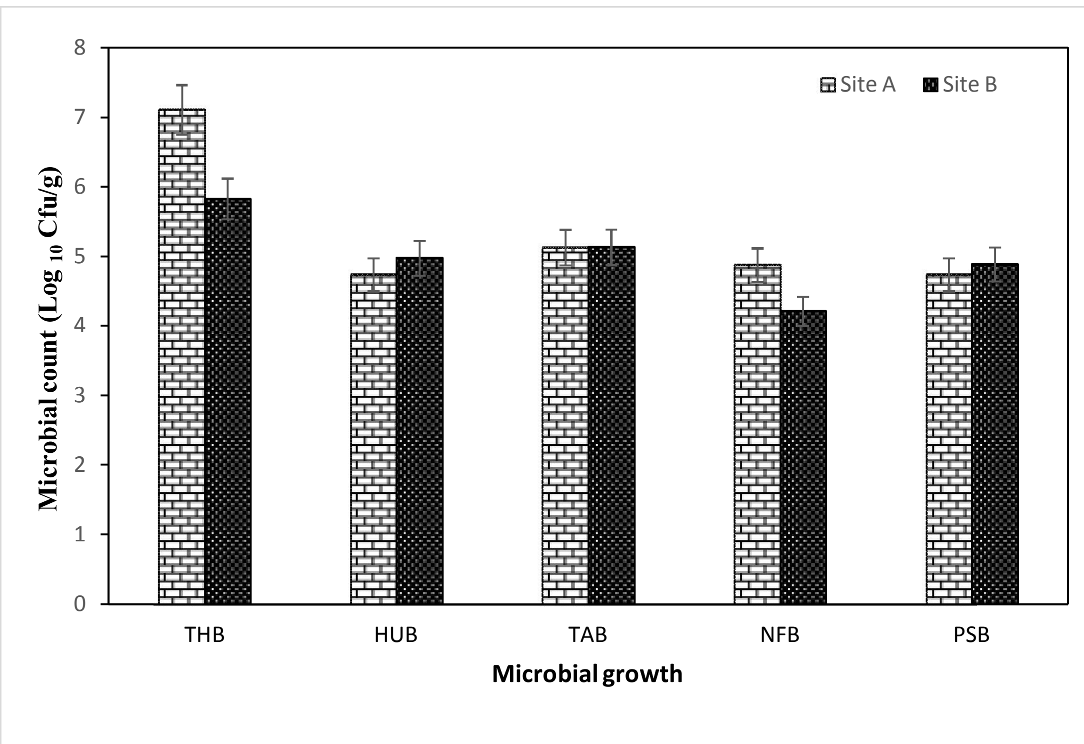

Fig. 1: Baseline characterization of crude oil polluted soil samples obtained K-Dere

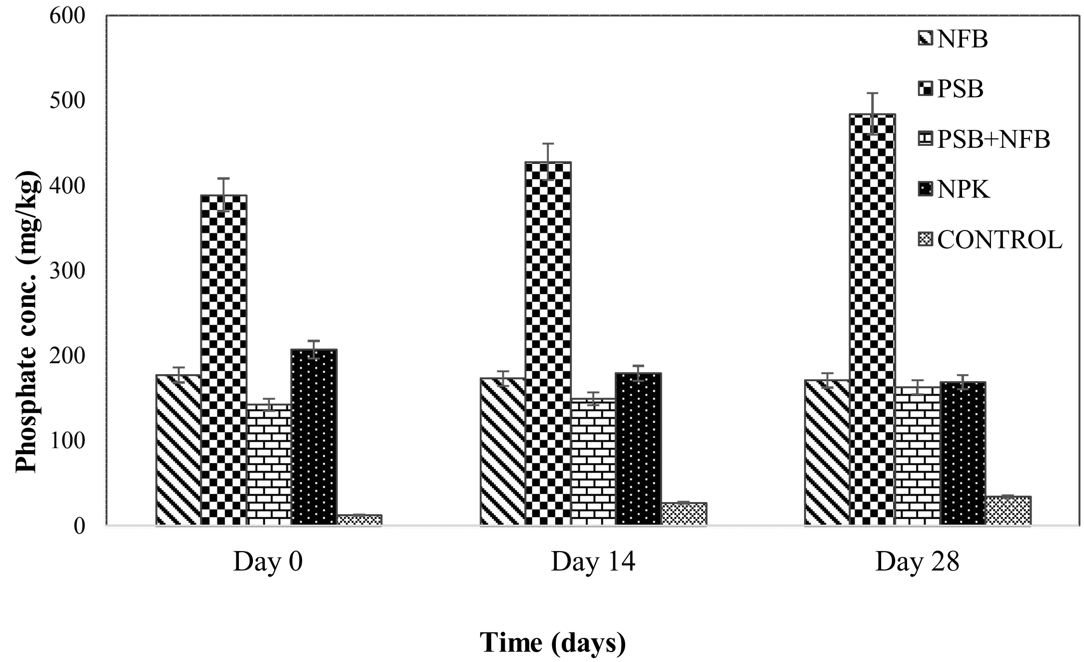

Fig. 2: Changes in phosphate level (mg/kg) in different treatment setups during 28-day

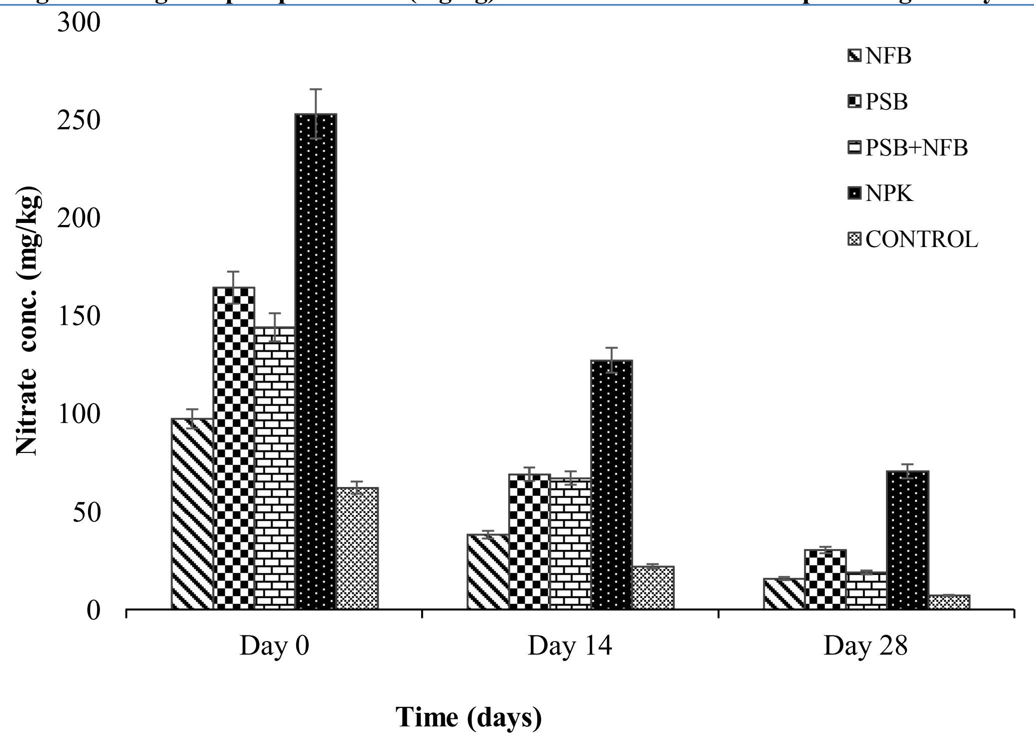

Fig. 3: Changes in nitrate (mg/kg) in various treatments during 28 day

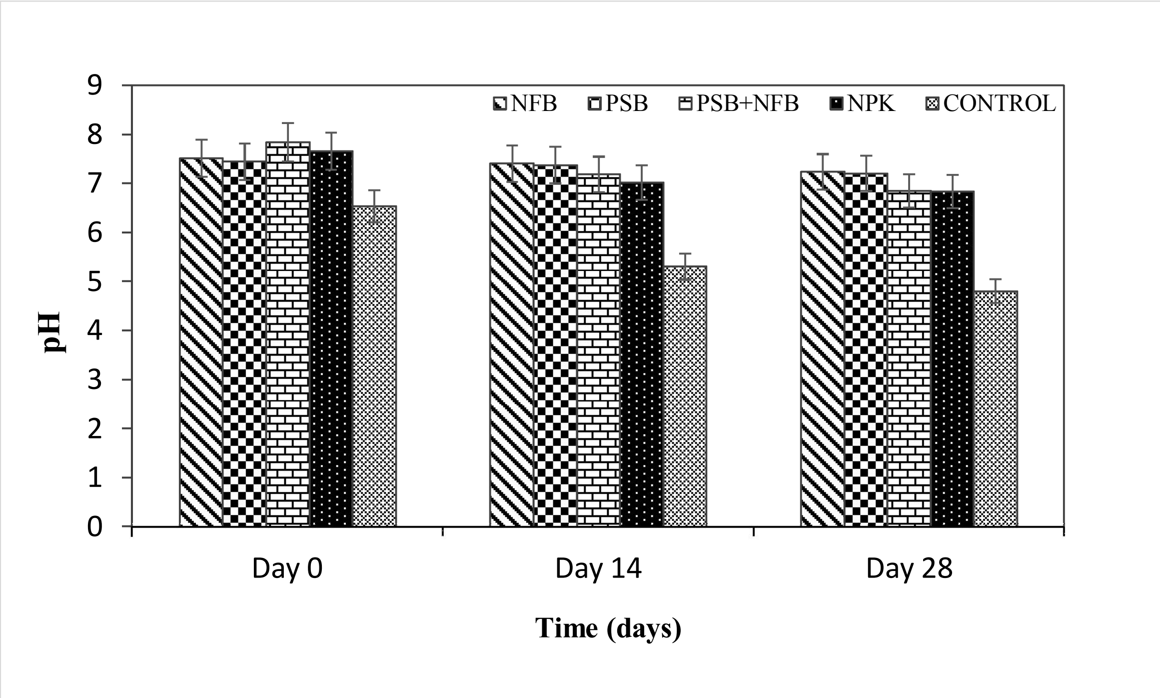

Fig. 4: Changes in pH in different treatment setups during 28-day study period

The results in Fig. 6 described alterations in the TOC. The treatments modified NFB slightly and decreased from 35.69 to 30.57 mg/kg on 28th day while PSB-amended sample declined from 36.08 to 31.09 mg/kg on day 28. The setups NFB+PSB and NPK-amended samples had TOC value of 28.82 mg/kg and 32.44 mg/kg on 28th day respectively. However, control had slight reduction from 26.71 mg/kg to 25.05mg/kg.

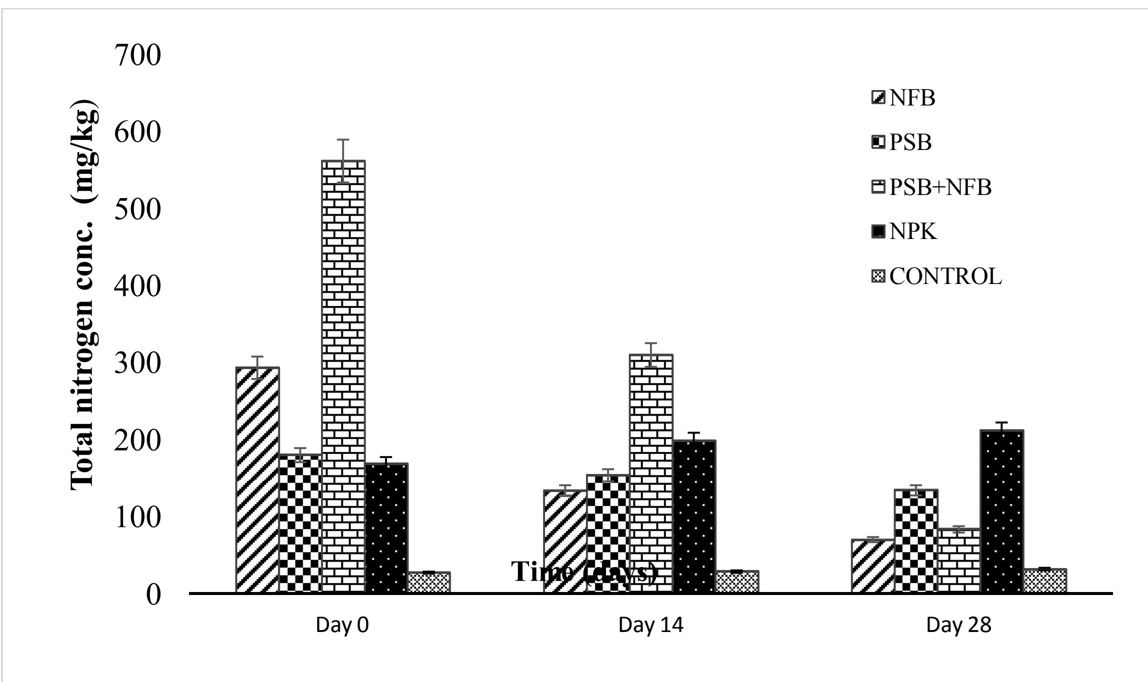

Fig. 5: Changes in Total Nitrogen (mg/L) in different treatment during 28-day study

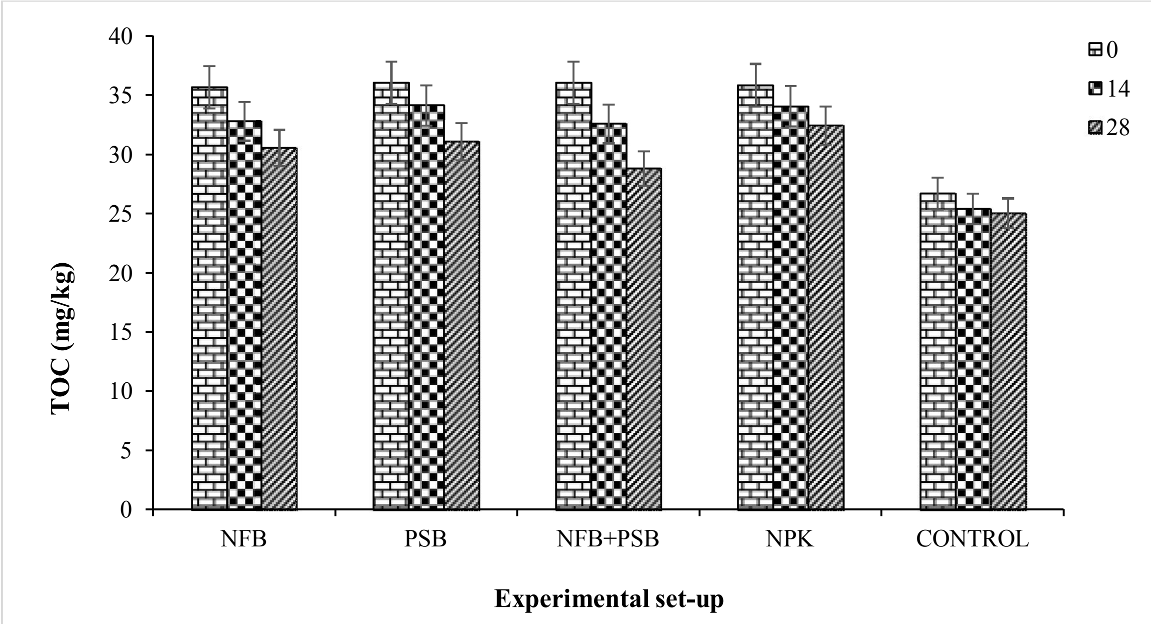

Fig. 6: Changes in TOC in different treatment setups during 28-day research period.

The result presented in Fig. 7 described alteration in bacterial population during bioremediation study. Notably, PSB-amended sample was observed to have increase in aerobic count from 8.1 to 9.5 and 9.6 Log_{10}cfu/g while NPK-amended sample was observed to have 7.05 Log_{10}cfu/g to 7.5Log_{10}cfu/g and finally to 8.79Log_{10}cfu/g for 0, 14 and 28 days respectively. Similarly, there was no particular trend in control set up as it is seen to increase from 6.4 to 6.404 on the 28th day of monitoring. The results presented in Fig. 8 revealed a slight increase in HUB count in setup amended with NFB from day 0 to day 14 to day 28 was 5.2, 6.9 and 5.0 Log_{10}cfu/g. The set up amended with NFB+ PSB had increase in microbial population from 4.72 to7.89 Log_{10}cfu/g on the 28th day. Set up amended with NPK revealed increase in bacterial population from 5.4 Log_{10}cfu/g to 6.8 Log_{10}cfu/g on 28th day.

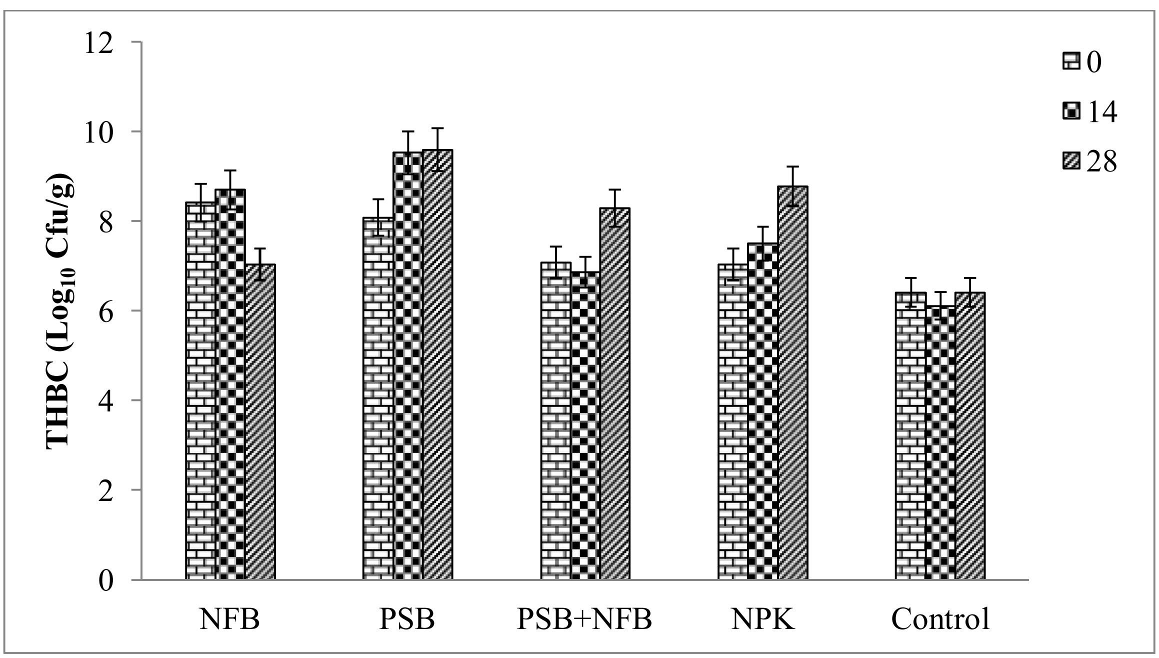

Fig. 7: Changes in THBC during bioremediation monitoring study

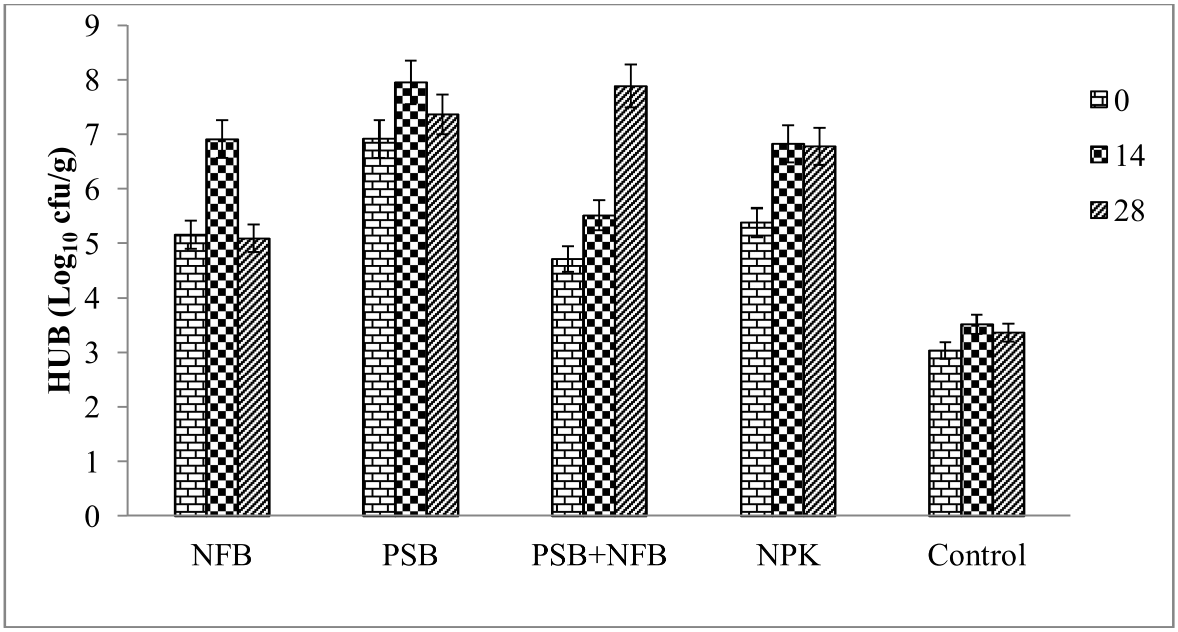

Figure 8: Changes in HUB counts during bioremediation monitoring study.

The results presented in Fig. 9 described the growth dynamics of phosphate-solubilizing bacterial flora during bioremediation. The result revealed that PSB amended set up showed sharp decrease from zero days to 28th day from 5.8 Log10 cfu/g to 5.17Log10 cfu/g. The set up modified with NPK had increase from 5.0 to 6.9 Log10 cfu/g. The result of control revealed that microbial load never showed appreciable trend between initial days to 28th day of this research. The results presented in Fig.10 shows the changes in nitrogen-fixing bacterial counts in the setups amended with NFB for day 0, 14 and 28 were 5.0, 6.5 and 5.2 Log$_{10}$cfu/g. The set up amended with NFB+PSB had increase in microbes population from 5.2 to 6.7 Log$_{10}$cfu/g on day 0 and 14 while a decrease was observed on the 28-day as 5.7 Log$_{10}$cfu/g. NPK amended set up had an increase from 5.0 to 6.0 Log10 cfu/g and later declined to 5.3 on 28th day. The result from control revealed that microbes load from initial day increased and slightly decreased before 28-day.

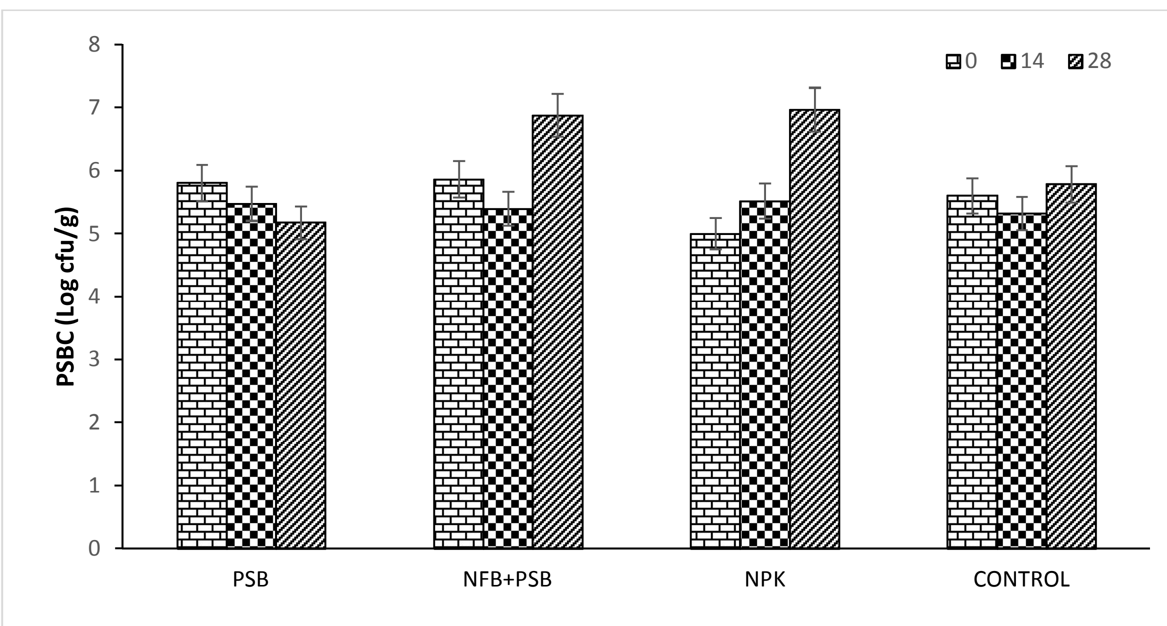

Fig. 9: Changes in PSB counts during remediation study

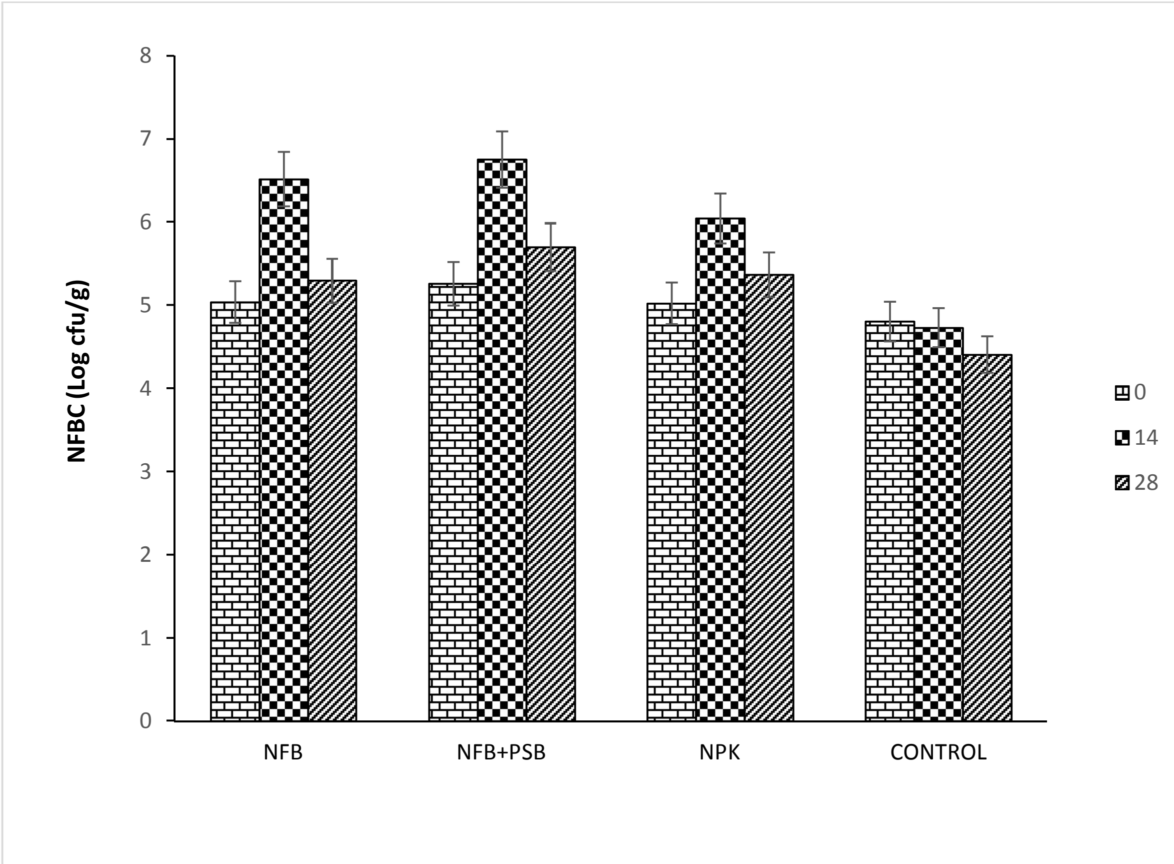

Fig. 10: Changes in NFB counts during remediation study

The TPH is presented in Fig. 11. The results from crude polluted soil modified using NFB bio-fertilizer suggest reduction in TPH from 8987.5742 mg/kg to 193.7225 mg/kg. Furthermore, samples treated with PSB bio-fertilizer alone reduced from 8987.5742 mg/kg to 511.1189 mg/kg, samples amended with NFB and PSB bio-fertilizers declined from 8987.5742 mg/kg to 226.7071 mg/kg. The NPK and control samples reduced from 8987.5742 mg/kg to 713.0582 mg/kg and 8987.5742 mg/kg to 5876.2402 mg/kg respectively.

The results presented in Fig. 12 describe the percentage removal of TPH in setups NFB and PSB had a reduction of 97.8% and 94.3% respectively. The NFB+PSB amended sample had TPH declined of 97.4% while NPK and control had decreased to 92.1% and 34.6% respectively. The results in Fig 13 described the growth characteristics of maize plants on different bio-fertilizer concentrations.

The treatments varied with different concentrations of remediated soil and biofertilizers (NFB, PSB and NFB+PSB). The result revealed that NFB treatment had the best seed germination from the initial day 6 to 12th day with concentration ratio of 25:75 with growth height of 20 and 32 cm respectively. Meanwhile, concentration of 100:0 had retarded growth from 8 to 15 cm on 12-day.

The PSB treatment revealed the seed growth in the concentration ratio of 25:75 observed the highest growth of 10 to 30 cm on the 12-day while the set-up concentration ratio of 100:0 was 18 cm to 23 cm respectively. The results for mixed NFB+ PSB treatment indicated that seed growth in the ratio of 25:75 showed highest growths from 18 to 31 cm while no seed growth was witnessed in100:0 concentration ratio.

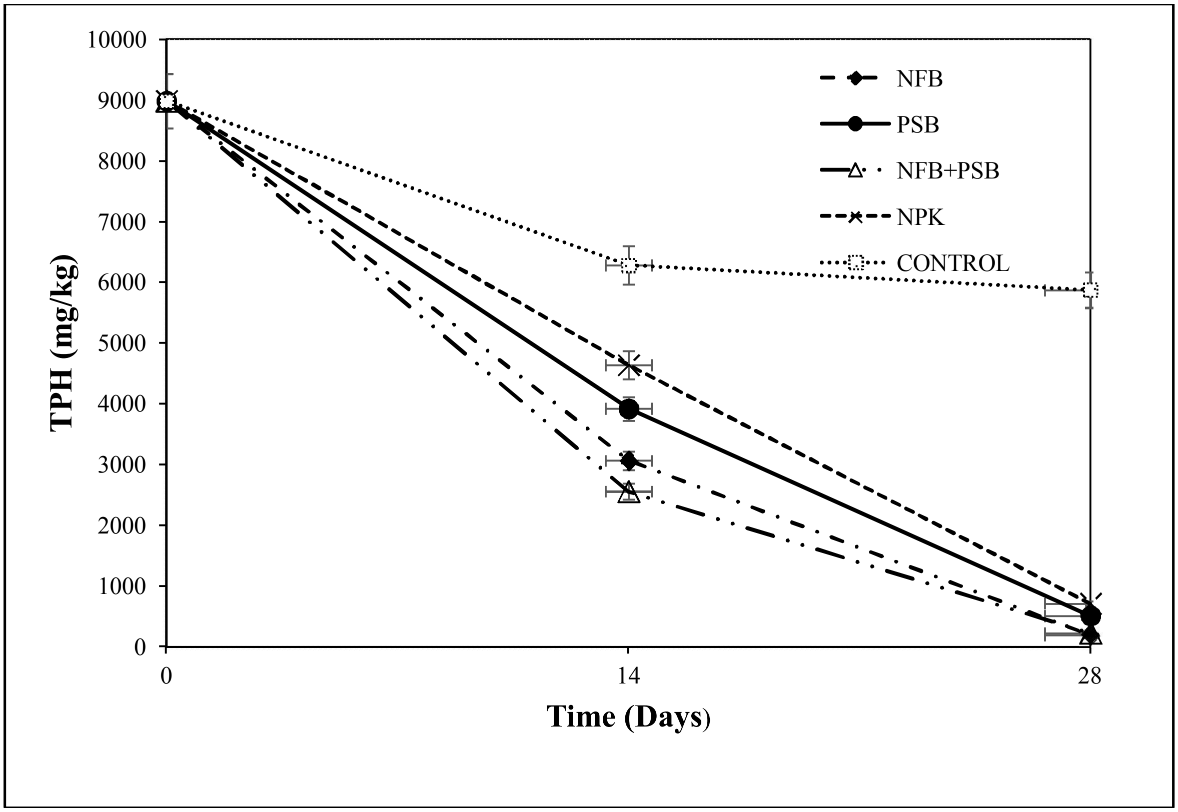

Fig. 11: Changes in TPH of soil from different treatments during research period

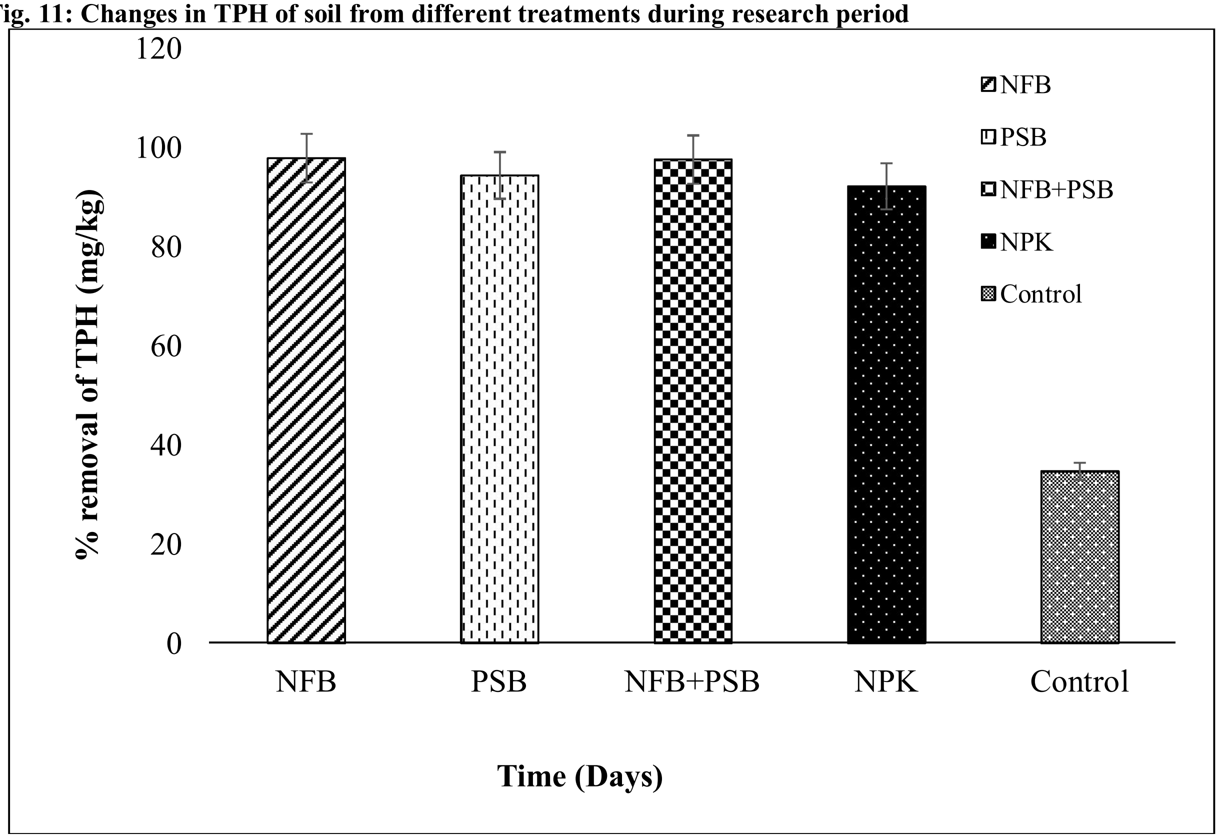

Fig. 12: Percentage removal of TPH from soil obtained from various treatments

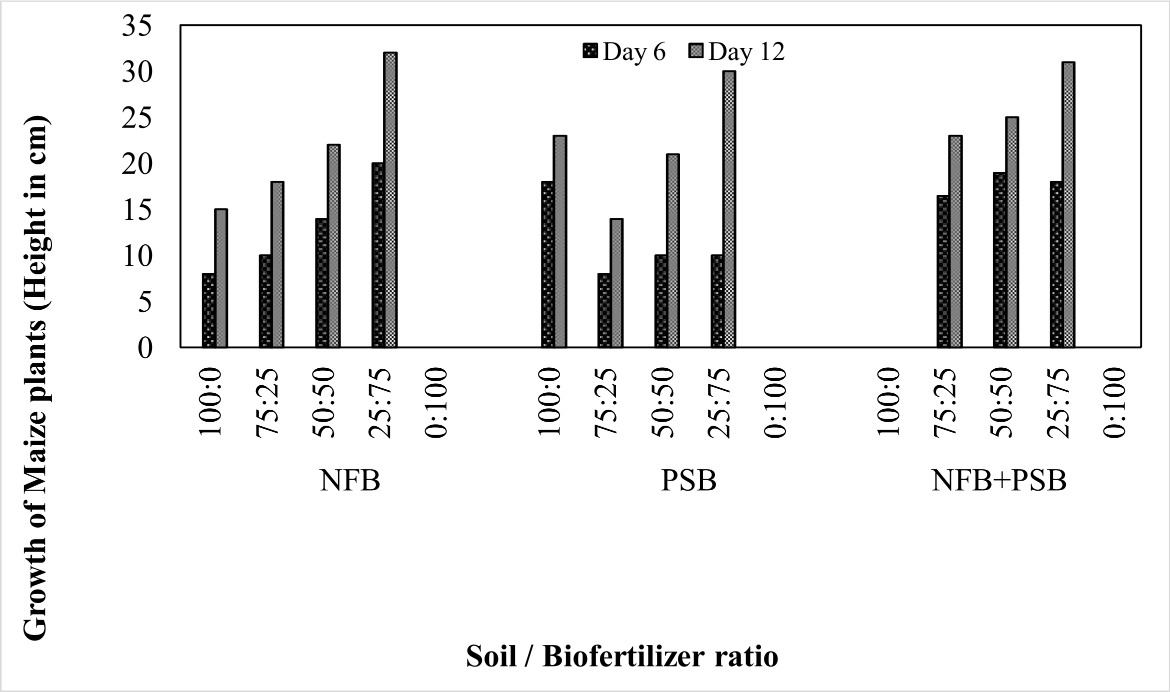

Fig 13: Growth characteristics of maize plants in different biofertilizer concentrations

The molecular characteristics of the bacterial isolates after being analysed on agarose gel electrophoresis are presented in Plate. The genes were amplified on the gel. Isolate with codes PSBGB-10, NFGB-8, PSBGB-4 and NFGB-11. The isolates were observed to have bend size of 1.6kbp on 16S universe primers. Isolate NFGB-8was observed to have a 99% with *Azotobacter* chroococcum. The PSBGB-10 was observed to have a 98% similarity with *Bacilllus firmus*. Lane 1: DNA maker; Lane 2 and 3: negative and positive controls; Lane 4 and 5: nitrogen-fixing bacteria; Lane 6 and 7: phosphate-solubilizing bacteria. *Primer set used*=27F (5[1]AGAGTTTGATCMTGGCTCAG-3[1]) and 1492R (3[1]GGGTTACCTTGTTACGACTT5[1]). Neighbour-combining phylogenetic tree of isolates NFB-8, NFB-11, PSB-4 and PSB-10 are presented in Fig. 14.

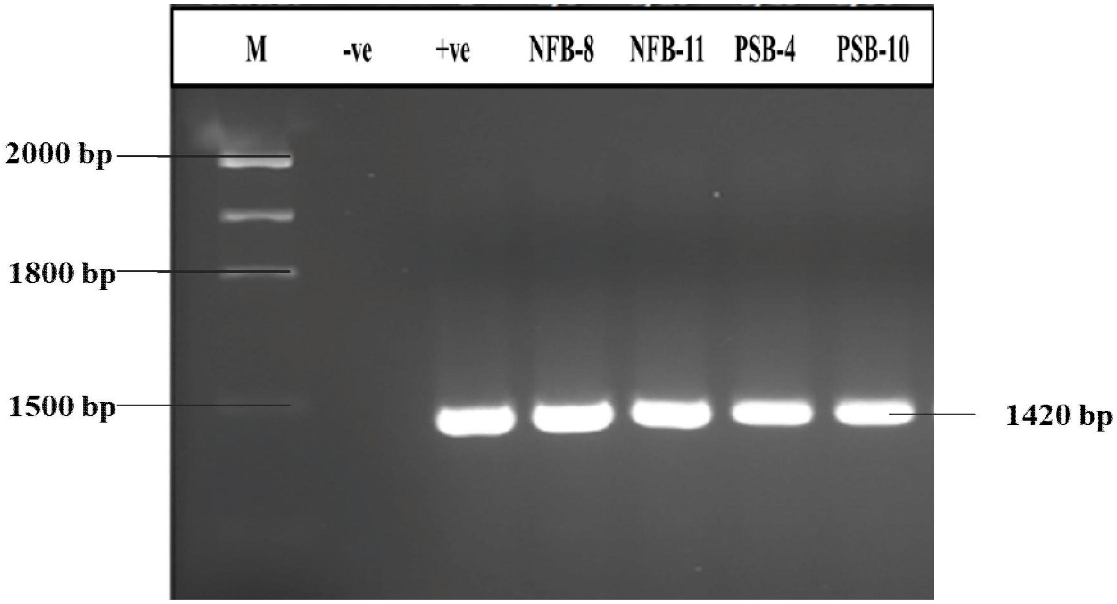

Plate 1: PCR amplification images of the 16S rRNA gene bands of the NFB and PSB.

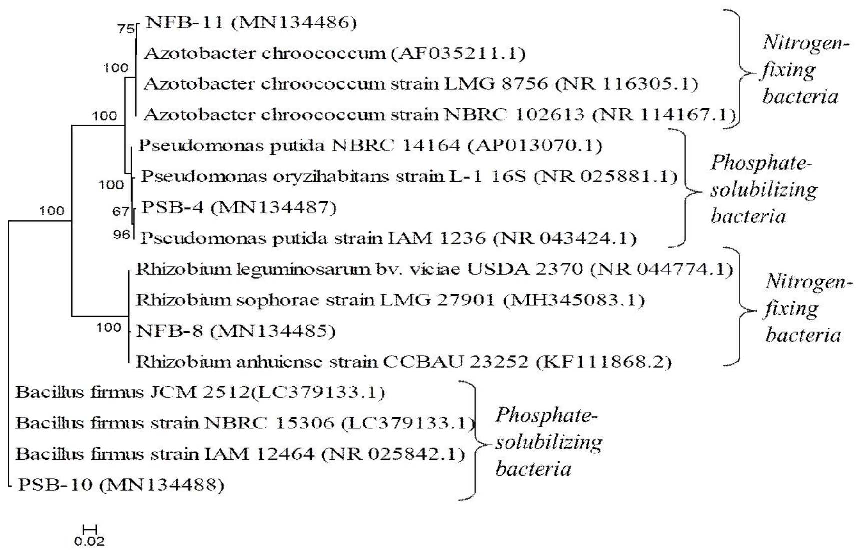

Fig. 14: Neighbour-combining phylogenetic tree of isolates NFB-8, NFB-11, PSB-4 and PSB-10 made by MEGA 6.0 [17]. Bootstrap values of >50% (based on 1500 replicates) are given in the nodes of the tree. Nucleotide substitution mode used was Jukes and Cantor. NCBI accession numbers are given in parentheses

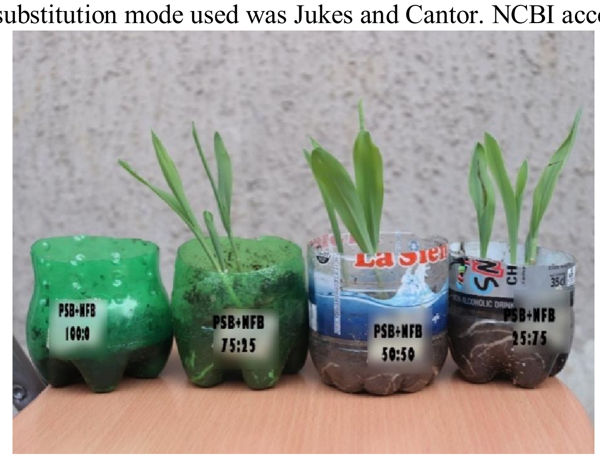

Plate 2: Cultivated maize plant using various concentrations of nitrogen- fixing and phosphate-solubilizing biofertilizer (day 6)

Plate 3:Cultivated maize plant growing best in higher concentration of nitrogen-fixing and phosphate-solubilizing biofertilizer (day 12)

Plate 4: Cultivated maize plant using different concentrations of phosphat solubilizing biofertilizer on day 6.

Plate 5: Cultivated maize plant growing best in higher concentration of phosphate solubilizing biofertilizer on day 12.

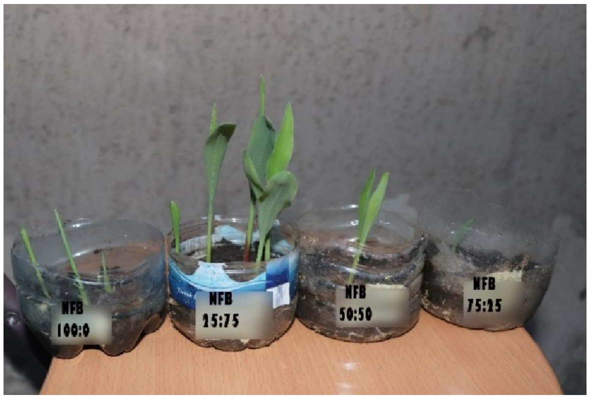

Plate 6: Cultivatied maize plant using different concentrations of nitrogen-fixing biofertilizer on day 6.

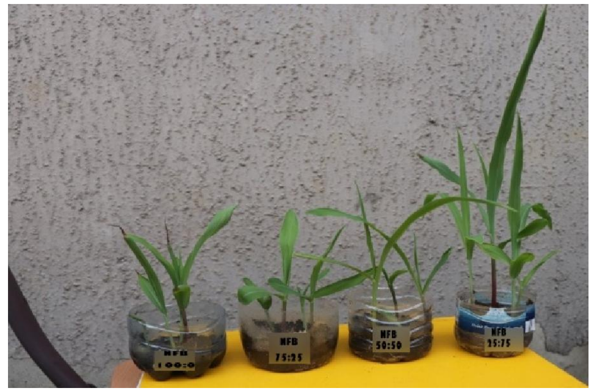

Plate 7: Cultivated maize plant growing best in higher concentration of nitrogen- fixing biofertilizer on day 12.

4. Discussion

In this study, bioremediation of crud polluted soil using bio-fertilizer from phosphate-solubilizing and nitrogen-fixing bacteria was examined. Five set ups were used with varying bio-stimulation conditions in crude polluted area. The basic physicochemical and microbiological features of crude polluted soil sampled was observed to have a pH of 6.5 ±0.169, temperature 28 ±0.6 ^{o}C moisture level of 0.16 ±0.01 %, Electrical conductivity 3.6 ±0.25 µs/cm, nitrate 86.2 ±0.35 mg/kg, phosphate 34.8 ±0.7 mg/kg, sulphate 24.1 ±0.5 mg/kg, TOC 14.18%.

The heterotrophic bacteria number of 1.58 x 10^7 ±0.205cfu/g, total fungal count of 3.9 x 10^6 ±0.004cfu/g, HUB number of 7.9 x 10^4±0.170cfu/g, nitrogen-fixing bacteria number 7.4 x 10^4±0.162 cfu/g and phosphate-solubilizing bacteria number 5.3 x 10^4±0.105 cfu/g (Table 1). These values suggest that crude polluted soil was loaded with physicochemical parameters and a vast microbial diversity. Furthermore, presence of these highly polluted soils can induce loss in viability of cells and reduction in microbe's population and thereby retarding indigenous flora which could thereby enhance the possible degradation of polluted soil. These values compared with findings from Orhorhoro *et al*. [18] who reported pH of 6.38 and moisture level 16.48. The pH of crude polluted area was slightly neutral, which suggest it could allow bacteria flora growth. These suggest that crude polluted area had variable acidity level.

The low water amount equally indicates poor bioavailability of crude components to microbial load. The TPH content of the soil was 8987.5742 ±0.00 mg/kg and THC of 6000 ±0.00 mg/kg suggest fraction of hydrocarbon were laden in polluted area as spent material, these corresponds with report of Ayotamuno *et al*. [19] who reported 69372 mg/kg, which is higher than result obtained from this study, the TPH content suggest high load of contaminants of health concern. Bacterial isolates utilized are identified as *Azotobacter* sp., *Rhizobium* sp., *Pseudomonas* sp. and *Bacillus* sp. using their morphological, bio-chemical and molecular characteristics.

These bacteria were formally reported as putative hydrocarbon mortifying bacteria [20, 21, 22]. The role of microorganisms in hydrocarbon dmortification is significant in contaminant removal and detoxification and this may be attributed to bacterial induced enzyme system and adaptation due to pre-exposure to hydrocarbon pollutants. THB count of polluted area in site A was 7.12 Log_{10} cf/g while sample obtained from site B was 5.82 Log_{10} cf/g. The HUB count obtained was 4.7 Log_{10} cf/g and 4.9 Log_{10} cf/g for site A and B respectively. Total aerobic bacterial count was fairly the same in both polluted soil samples 5.12 Log_{10} cf/g and 5.25 Log_{10} cf/g for site A and B. Shaman *et al*. [23] reported a count range of 8 x 10^3 – 4.8 x 10^8 cfu/g for bacteria count for polluted soil in bio-reactor set up. In related study, Nwogu *et al*. [24] reported 3.4 x 10^5 – 2.7 x 10^6. Nitrogen-fixing bacteria number was 4.87 and 4.21 Log_{10} cf/g for site A and B while phosphate-solubilizing bacteria number was 4.73 and 4.88 Log_{10} cf/g respectively.

Changes in nitrates and phosphate level which are key sources of ion needed for exchange of reducing agents and oxidizing agents were monitored throughout the study. The nitrate contents for day zero were 97.46, 164.18, 143.9, 252.91 and 62.17 mg/kg for the setups NFB, PSB, NFB+PSB, NPK and Control, respectively and after day 28, the values had decreased to 15.94, 30.43, 18.98, 70.53 and 7.30 mg/kg. By day 28, more rapid decline in nitrate content was observed across the bio-stimulated crude polluted soils. The phosphate content for day 0 were 177.5 mg/kg for NFB, 389.07 mg/kg for PSB, 142.41 mg/kg for NFB+PSB, 207.16 mg/kg for NPK and 12.22 mg/kg for the Control.

The highest phosphate content was observed at day 14. Similarly, Ogugbue *et al*. [10] reported increased nitrate and phosphate concentrations in experimental setups over a bioremediation study period and their report is similar to this findings. The reduction in nitrates and phosphates were significant after 2 weeks of this experiment. This outcome agree with Ayotamuno *et al*. [19] who suggested that decline level of nutrient supplement, may have stimulated response in increase in bacteria population. A PH change over this bioremediation research was monitored in different set-ups. PH condition affects nutrient availability. During this research, pH of soil modified with NFB changed from pH 7.5 to 7.4 and 7.2. This pH range is within the alkaline level reported by Nwogu *et al.* [24]. Meanwhile, pH in the NFB+PSB setup decreased from slightly alkaline to neutral throughout the study. These results agreed with the findings of Ogugbue *et al*. [10], who stated that shift in pH value in neutrality direction might be because of extinction of crude-based hydrocarbons present in polluted area since neutral level of pH is optimum for bacteria metabolism and growth. Neutral pH condition favored ability of used bacteria to degrade crude.

The pH in control setup reduced from 6.5 to 4.8. This concord with work of Ayotamuno *et al*. [19] who reported that control pH of set up declined during bio-remediation in crude polluted area. Changes in population density of different microbial groups in soil samples were determined by numbering of total cultivable heterotrophic bacteria and total cultivable hydrocarbon using bacteria as presented in Figs. 4.7 and 4.8. In set ups NFB and PSB, there was gradual increase in HUB population with time suggesting that cells utilized hydrocarbon as source of carbon and energy. The results also suggest that the gradual increase in THUB population might be linked to nutrient availability and subsequent decrease in number as nutrients were depleted.

For example, in setups NFB, the HUB population increased from 5.2$\log_{10}$ cfu/ml on zero days to 6.9 $\log_{10}$ cfu/ml on the day 14. Meanwhile the counts of THUB reduced to 5.0 $\log_{10}$ cfu/ml on the day 28. This reduction in THUB counts might be linked to decline in hydrocarbon/pollutants concentration (carbon and energy source). This decrease in microbial number and population after day 28 might be linked to nutrients depletion, complete crude oil metabolism as they were added once, and toxic metabolites accumulation. The results are in line with those obtained by Onifade and Abubaker [25], who reported a sharp reduction in microbial counts and attributed this decline to nutrient limitation. Furthermore, in the set ups NFB + PSB and NPK, the HUB population increased over time, might be because of nutrient availability/utilization as bio-stimulation. More so, in the NFB+PSB treatment population of HUB increased from 4.72 $\log_{10}$ cfu/ml on the day zero to 7.89 $\log_{10}$cfu/ml by day 28 and there was appreciable ($P<0.05$) rise in microbe population between day 0 and 28. The increase in population of HUB could be attributed to role of bio-stimulation and to utilizing nutrient by bacteria isolates [26]. The rapid growth of HUB in NFB, PSB, NFB+PSB and NPK might be linked to modified nutrients in treatment set-ups.

The nutrients which are particularly potassium, nitrogen, and phosphorus perhaps stimulated microbe's growth and permitted microbes to produce necessary and crucial enzymes needed to breakdown petroleum-based hydrocarbon contaminants since they are main foundation of life [27]. Although, HUB were present in polluted area their numbers might not be sufficient to commence effectual contaminated soil remediation. Therefore, activities of HUB are stimulated by supplying or inputting of carbon, nitrogen, and phosphorus which are used by these modifying microbes for their metabolic performance [28].

Previous research showed that nitrogen is crucial for cell-based protein and cell-wall formation while phosphorus is crucial for nucleic acids, ATP formation and cell membrane [29]. The bioremediation study involves phosphate-solubilizing bacteria shows increase phosphate concentration with proliferate increases in biomass of organism in NFB+PSB and NPK samples. The initial rise in soil available phosphorus was because of ability of organism to solubilize phosphate present in soil. According to Debojyotic *et al.* [6] that started that PSB are very crucial in solubilization to insoluble phosphate by release of organic acids. Moreover, the sample PSB reveals progressive decreases after initial day because of fact that they used hydrocarbon as metabolite in building biomass. The isolated organisms *Pseudomanas* and *Bacillus* spp. play active roles in bioremediation of crude polluted area by degrading hydrocarbon components. This result agrees with findings from Abu and Ogiji [13] and Zhu *et al*. [30] who showed that phosphate was used by microbes during bioremediation research.

It was established that phosphorus availability limits microbe's degradation for hydrocarbon. Chikere *et al.* [31] reported on ability of *Pseudomonas* sp. and *Bacillus* sp. to use crude as carbon source. They possess genes which code enzymes like catechol di-oxygenase, alkane monooxygenase and alkene sulfonate mono-oxygenase that help in breaking down hydrocarbon chains. The control sample in this research has no appreciable increase in microbial population which has linked to toxicity of crude oil components. During remediation on crude polluted soil, samples treated with nitrogen-fixing bacteria was noticed to have gradual reduction of hydrocarbon contents between day 14 and 28in nitrogen-fixing bacterial (NFB) bio-fertilizer from 6.2 Log_{10} cf/g to 5.2 Log_{10} cfu/g, NFB+PSB-bio-fertilizers from 6.7 Log_{10} cfu/g to 5.7 Log_{10} cf/g, NPK from 6.0 Log_{10} to 5.3 Log_{10} cf/g.

The isolated organisms *Azotobacter* sp. and *Rhizobium* sp. carried nitrogenase enzyme, capable to fix atmospheric nitrogen into the soil. According to Swain and Abhijita [32] both organisms act as bio-fertilizer in supplying macronutrient. This observation agrees with Ogugbue *et al*. [10] that started that hydrocarbon-based polluted soil with modified microbes like nitrogen-fixing bacteria aids in co-metabolism and nitrogen fixation to autochthonous bacteria which aid in availing needed nitrogen that would improve degradation of hydrocarbons. The findings of Agary and Ogunlaye [33] and Nwogu *et al*. [24] revealed that their numbers in soil are increased by bio-augmentation to speed up biodegradation rate. Biodegradation kinetics studies revealed that degradation efficiency for PSB-amended sample was 94.3% while NFB and NFB+PSB-amended plots were 97.8% and 97.5% respectively. However, the positive control was 92.1% while the negative control (Un-amended) was 34.6%. Similarly, the half-life of about 5 days was reported for NFB and NFB+PSB. PSB-alone had a half-life of 7days. Furthermore, NPK-amended set had a half-life of 8.0 days while the un-amended control had a half-life of 45 days.

In a related study Qin *et al.* [34] carried a similar work using biochar to deliver phosphates in a bioremediation and reported 84.8% loss in the TPH of the petroleum polluted soil. This report agrees with result from this study that nitrate and phosphates which deliver limiting nutrient to HUB community from exogenous sources could be functional in bio-removal of pollutants. In related research, Wu *et al*. [35] in a study where they employed bio-stimulation and bio-augmentation in bioremediation of crude polluted area reported 60% loss of TPH during their 40 day study duration.

This was in tandem with the report of this current research suggesting that delivering microbes cultures in development of bio-augmentation approach is pivotal to removal of TPH and contaminants of concerns [36]. The PAHs were observed to have reduced by 94.8% by the application of 50 g nitrogen-fixing bacterial biofertilizer. Other biofertilizers PSB and NFB+PSB were weighed 50 g also declined the PAHs to 96.0 and 98.1%. There is sharp increase in degradation of the PAHs in the three setups. The control only decreased by 25.3% component like benzene, 1, 2, 3- trimethyl-, naphthalene, anthracene, pyrene, benz (a) anthracene. This finding agrees with the results of Sutherland *et al*. [37] who reported presence of 1-methyl naphthalene, phenonthrene, fluorenthene, pyrene, chrysene, 2-methyl naphthalene, acenepthylene, acenephthene, fluorane, anthraene, benzo (a) anthraene, benzo (b) fluorenthene, benzo (k) fluorenthene, benzo (a) pyrene and indanol (1,2,3-cd)pyrene. At the end of day 14, NFB: diben (a,h) anthracene, indeno (1,2,3-cd) pyrene, benzo (g, h, i) perylene, PSB: benzo (k) fluoranthene, benzo (a) pyrene, diben (a, h)anthracene, indeno (1,2,3-cd) pyrene, benzo (a) pyrene, benzo (g, h, i) perylene. NFB+PSB: benzo (k) fluoranthene, benzo (a) pyrene, diben (a, h) anthrecene, indano (1,2,3-cd) pyrene, benzo (g, h, i) perylene.

The toxicity of the biofertilizer on routinely grown crop such as maize was studied. The toxicity testing involved 5 set-ups with ratios of remediated soil to biofertilizer of 0:100, 25:75, 50:50, 75:25, and 100:0, and these were performed for the different treatments. There was no growth of maize seeds planted in NFB+PSB in concentration of 100:0 while growth was observed for same concentration in NFB and PSB setups. Luxuriant growth of maize plants were recorded in 25:75 ratios for all the setups. This indicated that adequate nutrients were available for growth of maize seeds and that at 75% concentration; the bio-fertilizer was non-toxic to the maize seedlings.

The healthy growth is linked to uptake of crucial nutrients through well-developed root system and manifest on leaves as earlier reported [38-40]. The concentration of 0:100 bio-fertilizers in all setups (NFB, PSB, NFB+PSB) recorded no growth of seeds. The rate of moisture level cause asphyxiation of seeds and clogging of bio-fertilizer air pores to prevent growth. Applying bio-fertilizer for agricultural practices reduces risk of toxicity to environment. Odokuma and Ibor [5] reported that planting of the seed at once with fertilizer application may have accounted for absence of growth. This is expected since traditionally fertilizers are applied to farmlands weeks and months before planting [41].

5. Conclusion

Bio-stimulation of indigenous microbial communities was achieved by nutrient amendment strategies. The remediation outcome revealed that the most target limits were achieved within four weeks of the study. Total petroleum hydrocarbon and hydrocarbon contents were reduced to 97.8%. These were achieved by amending the polluted soil with nitrogen-fixing and phosphate-solubilizing bio-fertilizers. Phosphate, nitrogen and nitrate were reduced during the study using the 50 g of the substrates.

The pH of the soil matrix was buffered by the nutrient amendment with both nitrogen-fixing and phosphate-solubilizing bio-fertilizers. This work shows that the bio-fertilizer contributed to greater percentage reduction of crude-based hydrocarbon. This occurred when nitrogen-fixing bacteria bio-fertilizer and combination of nitrogen-fixing and phosphate-solubilizing bacterial bio-fertilizer were used. The use of bio-fertilizer provides essential limiting nutrients in enhancing bioremediation on crude oil polluted area in K-Dere. The concentration ratio of 25:75 (remediated soil / bio-fertilizer) supports cultivation practices due to the non-toxicity of the biofertilizer.

5.1. Recommendations

Government agencies must be heartened to sponsor field executions for application of bio-fertilizer made with nitrogen-fixing and phosphate-solubilizing bacteria. Bio-fertilizers from nitrogen-fixing and phosphate-solubilizing bacteria should be provided to farmers for agricultural practices instead of chemical fertilizers. The nitrogen-fixing and phosphate-solubilizing acteria (*Azotobacter chroococcum, Rhizobium leguminosarum, Bacillus firmus* and *Pseudomonas putida*) isolated in this study when used for crude oil polluted soil amendment, will stimulate indigenous hydrocarbon degraders to utilize hydrocarbon.

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